Neuron, Vol. 14, 467–475, February, 1995, Copyright © 1995 by Cell Press

The Precursor Protein of Non-Aβ Component of Alzheimer's Disease Amyloid Is a Presynaptic Protein of the Central Nervous System

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Summary

Non-Aβ component of Alzheimer's disease amyloid (NAC) is the second component in the amyloid from brain tissue of patients affected with Alzheimer's disease. Its precursor protein (NACP) was shown to be a brain-specific protein. In rat brain, NACP was more abundant in the neocortex, hippocampus, olfactory bulb, striatum, thalamus, and cerebellum and less abundant in the brain stem. Confocal laser microscopy analysis revealed that anti-NACP immunostaining was colocalized with synaptophysin-immunoreactive presynaptic terminals. Ultrastructural analysis showed that NACP immunoreactivity was associated with synaptic vesicles. NACP sequence showed 95% identity with that of rat synuclein 1, a synaptic/nuclear protein previously identified in rat brain, and good homology with Torpedo synuclein from the electric organ synapse and bovine phosphoneuroprotein 14 (PNP-14), a brain-specific protein present in synapses. Therefore, NACP is a synaptic protein, suggesting that synaptic aberration observed in senile plaques might be involved in amyloidogenesis in Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by neuronal loss and gliosis, which are accompanied by amyloid deposits at the core of senile plaques and on the vessel walls (Joachim and Selkoe, 1992; Price et al., 1992). A 39–43 amino acid polypeptide, Aβ, was found as a component of amyloid in AD (Glenner and Wong, 1984; Masters et al., 1985; Mori et al., 1992). Recent immunohistochemical studies have shown that, in addition to Aβ, many other proteins are associated with AD amyloid, including α1-antichymotrypsin (Abraham et al., 1988), apolipoprotein E (Namba et al., 1991; Strittmatter et al., 1993), complement proteins (Ishii and Haga, 1992), serum amyloid P (Duong et al., 1989), SP-40,40 (Choi-Miura et al., 1992), heparan sulfate proteoglycan (Snow et al., 1988), immunoglobulins (Ishii et al., 1975), gamma-trace peptide (Vinters et al., 1990), and lysosomal proteinases (Cataldo et al., 1990, 1994).

Recently, the Non-Aβ component of AD amyloid, NAC, consisting of at least 35 amino acids, was found in the amyloid-enriched fraction of AD brain, and antisera raised against NAC were found to stain both senile plaque amyloid and vessel amyloid. NAC, along with Aβ, was copurified with amyloid in the SDS-insoluble fraction and sequenced biochemically, which makes NAC distinct from other amyloid-associated components that are detected only by immunological methods, suggesting that NAC may be tightly associated with Aβ and may have a role in amyloidogenesis in AD brain. A cDNA encoding the precursor protein of NAC (NACP) was cloned (Uéda et al., 1993), and it was found that NACP is composed of 140 amino acids. NACP is recovered in the cytosolic fraction of human brain homogenate as a protein with an apparent molecular mass of 19,000 Da. NACP has seven repeated KTKEGV motifs but no signal peptide sequence nor N-linked glycosylation sites. NAC is located in the most hydrophobic portion of the NACP molecule. To evaluate further the role of NAC and NACP in AD pathogenesis, the investigation of physiological function of NACP is required. As a first step toward this effort, we localized NACP using immunohistochemical techniques. In this article, we show that NACP is located in presynaptic terminals in brain. Because the precursor protein of Aβ, APP, is also a presynaptic protein, our finding indicates the participation of two presynaptic proteins in amyloid formation in AD and raises the possibility that presynaptic pathology in senile plaques may be involved in amyloidogenesis in AD.

Results

Specificity of Antibody in Western Blot

Both antibodies, anti-NACP(1–9) against the NACP N-terminus and anti-NACP(131–140) against NACP C-terminus (Figure 1A), recognized a unique doublet band with molecular mass around 19,000 Da, located mainly in the cytosolic fraction of human and rat brain homogenates (Figure 1B). The lower band of this NACP doublet comigrated with recombinant NACP made in Escherichia coli. Comparison of the intensity of NACP staining in brain homogenates and the recombinant NACP preparation showed that as much as 0.5%–1.0% of the cytosolic protein was NACP. The staining of each antibody was abolished by incubating the antibody with its corresponding peptide (Figure 1B). Under the same experimental conditions, essentially all synaptophysin immunoreactivity was recovered in the particulate fractions, demonstrating that all synaptic vesicles were in the particulate fractions. Longer exposure of anti-NACP-stained blots showed faint NACP bands in the particulate fractions, leaving the possibility that a small fraction of NACP proteins may be associated with the particulate fractions.

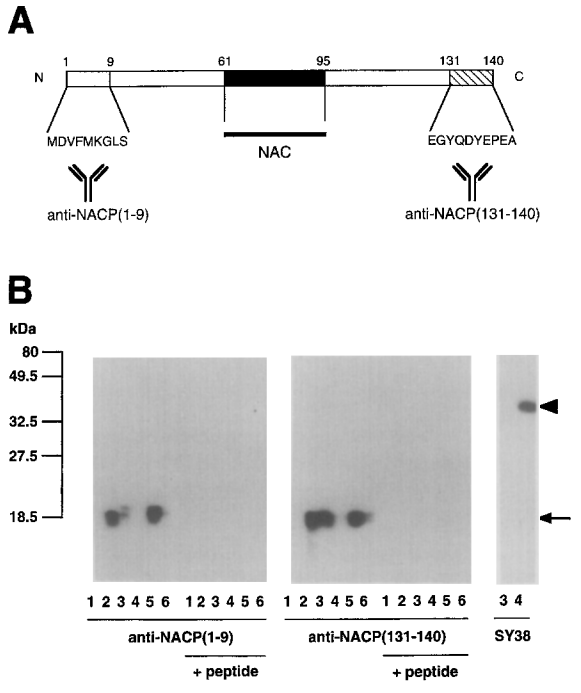


Figure 1. Specificity of Anti-NACP Antibodies

(A) Schematic presentation of NACP and its subdomain used to raise the antisera, anti-NACP(1–9) and anti-NACP(131–140). The NAC fragment (35 amino acids) originally found in the SDS-insoluble amyloid-enriched fraction of AD brain tissue is located in the middle of NACP (61–95).

(B) Western blot of cytosolic and particulate fractions from the homogenate of human brain, rat brain, and E. coli expressing human NACP. Lane 1, vector control E. coli homogenate; lane 2, NACP expressing E. coli homogenate; lane 3, cytosolic fraction of human brain; lane 4, particulate fraction of human brain; lane 5, cytosolic fraction of rat brain; lane 6, particulate fraction of rat brain. Anti-NACP(1–9) and anti-NACP(131–140) labeled the 19,000 Da doublet (arrow) mainly in cytosolic fractions. These immunoreactive bands were lost with preabsorption by the corresponding peptide (+ peptide). Anti-synaptophysin antibody, SY38, labeled the 38,000 Da protein (arrowhead) in the particulate fraction.

NACP Is Localized in the Presynaptic Terminals

Using the streptavidin–biotin–peroxidase (SAB) staining method, rat brain sections were immunostained by anti-NACP(131–140) and anti-NACP(1–9) antibodies. The intensity of immunostaining with anti-NACP(131–140) was relatively stronger in the cerebral cortex (layers II, III, and V), olfactory region, caudoputamen, nucleus accumbens, hippocampus, and cerebellar cortex than in the thalamus and brain stem (Figure 2A). The immunostaining pattern of the brain using anti-NACP(1–9) antibody was indistinguishable from that of anti-NACP(131–140) (data not shown). Immunolabeling was abolished by preincubation of the antibodies with the corresponding peptide (data not shown).

Throughout the various cortical and subcortical areas of the rat brain, anti-NACP immunostained the neuropil in a characteristic punctate pattern (Figures 2B–2D). Neuronal cell bodies, glial cells, and blood vessels were not immunostained. In the cerebral cortex, hippocampus, and cerebellar cortex, anti-NACP immunostained the neuropil following laminar patterns of arrangement (Figures 2A–2D). Ultrastructural analysis of sections immunolabeled with NACP confirmed the synaptic localization of this protein and showed that NACP was in close vicinity of, or in association with, the clear synaptic vesicles (Figures 2E and 2F).

Laser scanning confocal microscopic analysis of sections double immunolabeled with antibodies against NACP and synaptophysin showed that both markers colocalized in the great majority of the presynaptic terminals (Figure 3; Figure 4). In the neocortex, hippocampus, basal ganglia, olfactory region, and thalamus, between 70% and 100% of the axosomatic, axoaxonic, and axodendritic terminals contained both markers (Figure 5). In contrast, in the deep cerebellar and brain stem nuclei, although synaptophysin and NACP colocalization in axodendritic terminals was relatively common, only a small proportion of the axosomatic terminals contained NACP (Figure 3; Figure 4; Figure 5). In both cortical and subcortical regions, a small proportion of terminals was synaptophysin positive and NACP negative (Figure 4D–4I; Figure 5). Occasionally, punctate structures displayed NACP immunoreactivity but not synaptophysin immunoreactivity. Serial section analysis showed that these neuritic structures actually corresponded to the terminal segments of axons. Abundant NACP immunoreactivity was observed associated with specialized synaptic complexes, including the glomeruli of the olfactory bulb (Figure 3E), the glomeruli of the cerebellar granular layer (Figure 3H), and the mossy fiber synapses (Figure 4B).

Quantification of NACP and Synaptophysin

NACP protein was detected as a 19,000 Da doublet on an immunoblot of the cytosolic fraction of brain homogenate (Figure 1B). Synaptophysin protein bands were detected at 38,000 Da in the particulate fraction of brain sample by a mouse monoclonal antibody, SY38 (Figure 1B). Distribution of each protein was shown as a relative amount, with the amount in the frontal cortex set at 1. As shown in Figure 6, consistent with immunohistochemical results shown in Figure 2A and data of quantitation of NACP synapses shown in Figure 5, the concentration of NACP was high in the telencephalon (end-brain regions, including the olfactory bulb, frontal cortex, striatum, and hippocampus), intermediate in the hypothalamus and thalamus, and low in the midbrain, cerebellum, pons, medulla oblongata, and pituitary. Synaptophysin was more evenly distributed in most regions of the brain. However, the amount of this protein in the pituitary was small.

NACP Homologies

Computer search of the DNA sequence database (EMBL/GenBank Libraries) revealed that some proteins had sequences homologous to NACP (Figure 7). A search in peptide sequence databases for homologous sequences of the deduced 140 amino acid precursor primary sequence was performed at NCBI (National Center for Biotechnology Information, Bethesda, MD) using the BLAST network service (Altschul et al., 1990). NACP was found to be homologous to synucleins from human brain (Jakes et al., 1994),

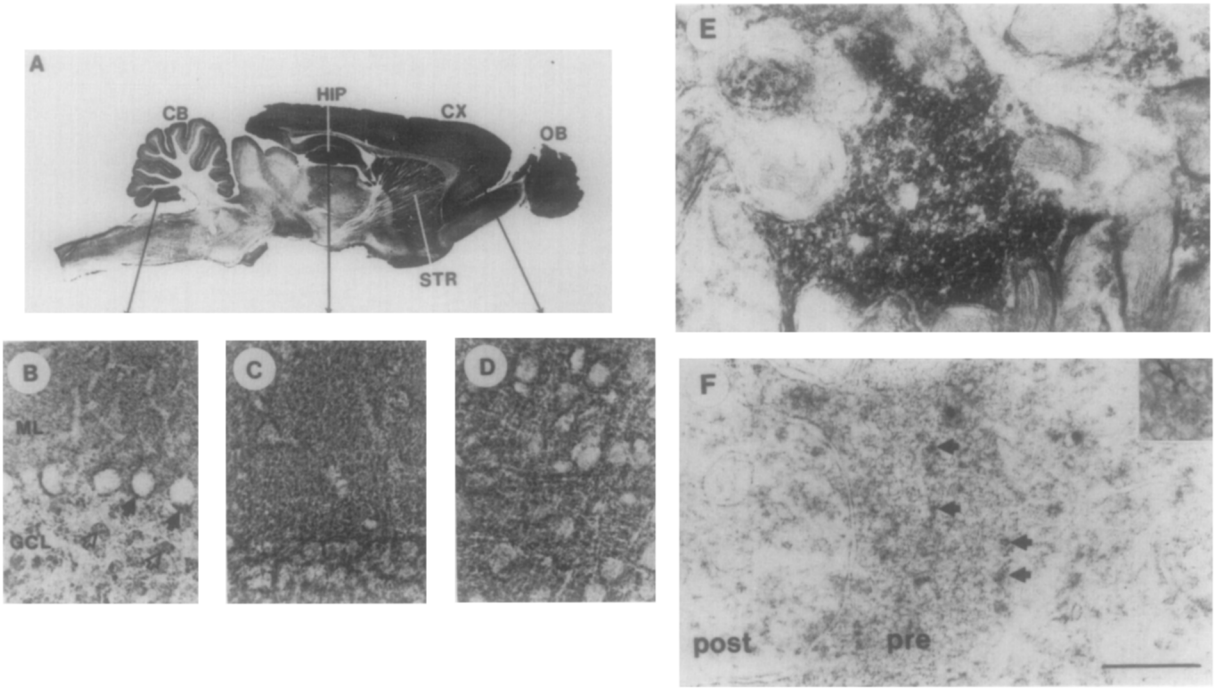


Figure 2. Distribution of NACP in Rat Brain Detected by Anti-NACP(131–140)

(A–D) Low magnification view of a sagittal brain section (A) shows that NACP immunoreactivity detected by the streptavidin-biotin-peroxidase (SAB) method was relatively strong in the neocortex, olfactory region, caudoputamen, hippocampus, and cerebellar cortex compared with the thalamus and brain stem. Higher magnification analysis showed a characteristic granular immunoreactivity throughout the brain, including the cerebellar cortex (B), the molecular layer of the hippocampal dentate gyrus (C), and the cerebral cortex (D). Closed arrows in (B) indicate axosomatic synapses around Purkinje cells, and open arrows indicate glomerular complex. CB, cerebellum; CX, cerebral cortex; GCL, granular cell layer; HIP, hippocampus; ML, molecular layer; OB, olfactory bulb; STR, striatum.

(E and F) Electron micrographs showing the localization of NACP in presynaptic terminals.

(E) Preembedding NACP immunostaining detected by DAB. The staining was confined to presynaptic terminals. (F) Ultrathin sections from the hippocampal CA3 region were immunolabeled with anti-NACP(131–140) followed by 5 nm gold anti-rabbit IgG. Gold-labeled NACP (arrows) was detected on or close to presynaptic vesicles. The inset is a higher magnification to show the localization of gold (arrow) on presynaptic vesicles. Bar, 0.5 μm (E and F).

rat brain (Maroteaux and Scheller, 1991), and the electric organ synapse of Torpedo californica, the Pacific electric ray (Maroteaux et al., 1988), and to bovine phosphoneuroprotein 14 (PNP-14), a brain-specific protein present in synapses around neurons but not in glial cells and Purkinje cell bodies (Nakajo et al., 1993). These small, acidic, brain-specific proteins have common repetitive sequence motifs and similar hydrophobic profiles. NACP shares a 95% identity with rat synuclein, form 1 (SYN1); of the seven mismatches, three are conservative. As with SYN1, synuclein β (synβ), and PNP-14, NACP is essentially hydrophilic, with the exception of the hydrophobic NAC region in the center of the molecule. Common to their N-terminal halves are the repeat motifs, KTKEGV (Maroteaux et al., 1988), of which seven units can be identified in NACP. All prolines of these proteins are contained in their C-terminal regions, which are also rich in glutamic acid residues. The latter contribute to their acidity. Arginine, cysteine, and tryptophan are notably absent in all these proteins. Rat SYN3 and human EST01420, identified by random sequencing of human brain cDNAs (Adams et al., 1992), had homologous sequence to the N-terminal portion of NACP, but neither of them included the NAC portion. Homo sapiens putatively transcribed partial sequence (HSPTPS), found by the MRC Human Genome Mapping Project, had a homologous sequence corresponding to the NAC portion of NACP.

Discussion

To investigate the distribution of NACP found as a precursor of the second major amyloid component in AD, two antibodies recognizing the N- and C-terminals of NACP were prepared. Both showed specificity in Western blot and immunohistochemical studies. These antibodies against human NACP recognized rat NACP comigrating at the same molecular mass as human NACP of 19,000 Da, indicating that the N- and C-terminals are both conserved in these species. In Western blots, the protein band of human and rat NACP showed doublet bands, although bacteria-expressed NACP protein showed only a single band with a faint tailing of degraded products. It is conceivable that in eukaryotic cells NACP may undergo posttranslational modification, such as phosphorylation, as was suggested for PNP-14 (Nakajo et al., 1993; Shibayama-Imazu et al., 1993), for NACP has a PKC target motif (KTKEGV) seven times within its sequence (Kennelly and Krebs, 1991). It is also possible that NACP is a member

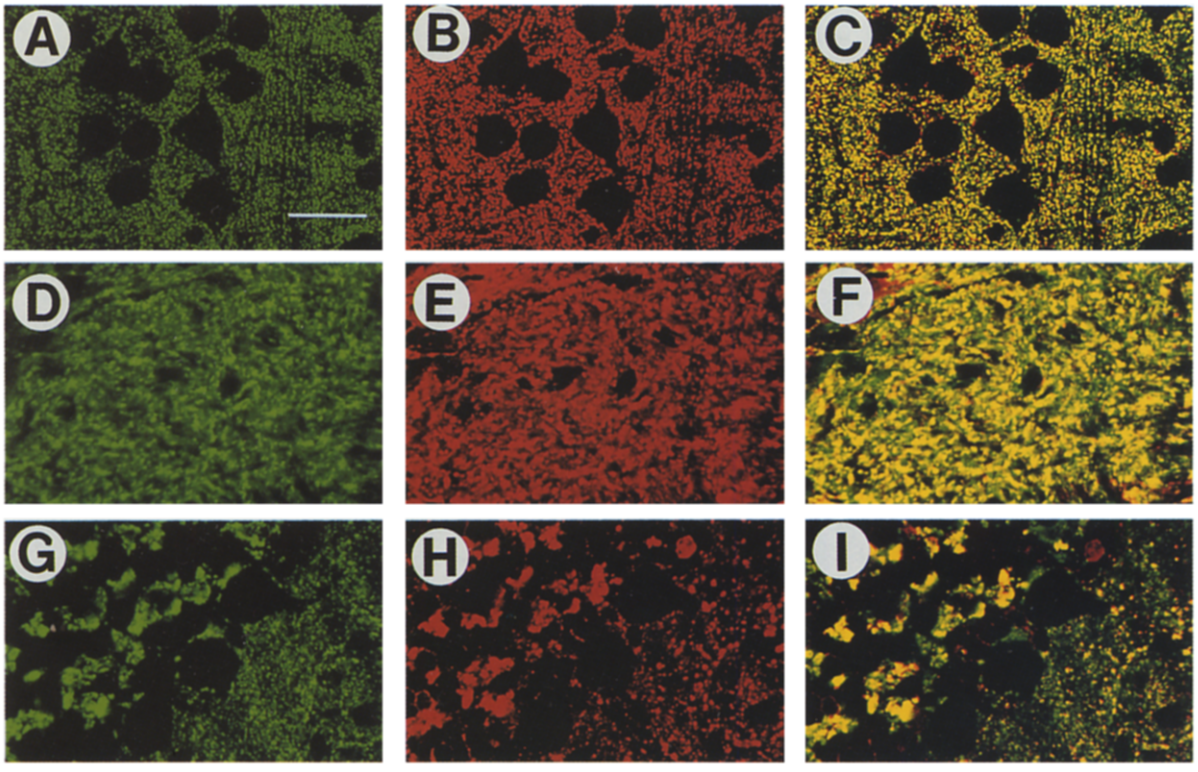
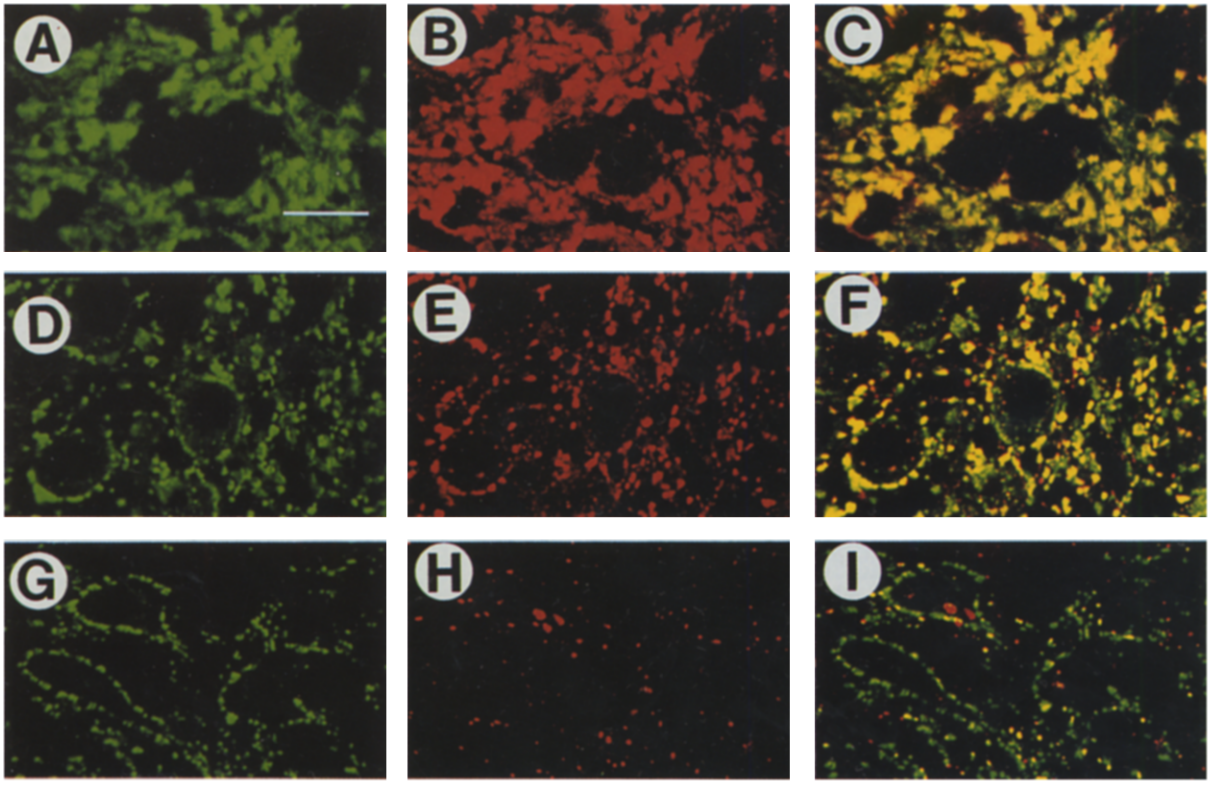


Figure 3. Laser Scanning Confocal Microscopy of Sections Double-Labeled for SY38 and NACP(131–140)

Mouse monoclonal anti-synaptophysin (SY38) is labeled with fluorescein isothiocyanate and shown in green (A, D, and G); NACP(131–140) is labeled with Texas red (B, E, and H). (C), (F), and (I) correspond to the electronically merged image; colocalization of NACP with synaptophysin is represented in yellow. (A–C) Neocortex; (D–F) glomeruli of the olfactory bulb; (G–I) cerebellar cortex. Bar, 15 μm.



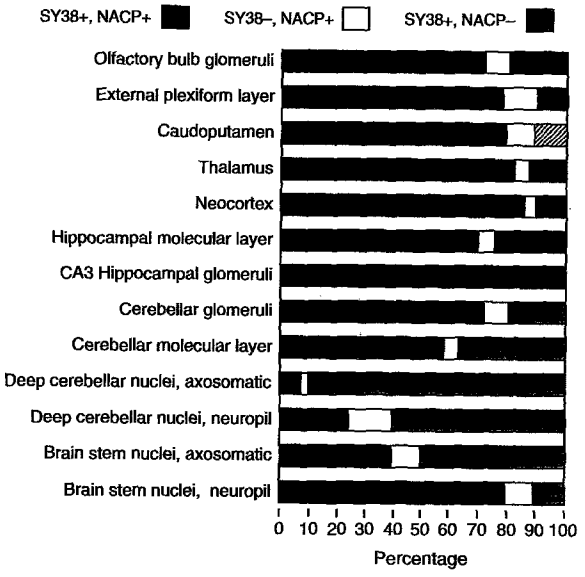


Figure 5. Computer-Aided Quantitation of Colocalization of NACP with Synaptophysin in the Presynaptic Terminals

In the cortical regions, a large percentage of the synaptophysin-immunoreactive terminals contained NACP. In contrast, in subcortical regions, a lower proportion of the synaptophysin-immunolabeled axosomatic nerve terminals contained NACP.

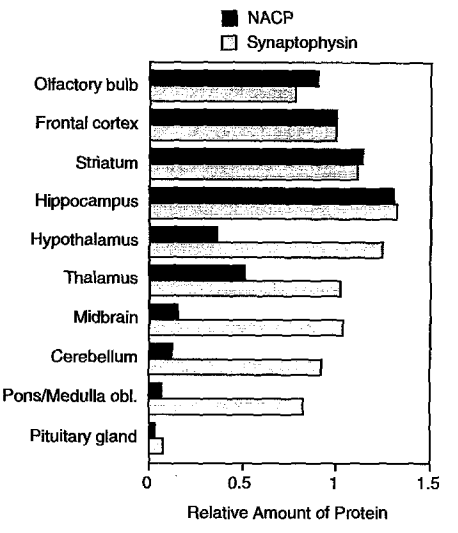


Figure 6. Western Blot Quantitation of NACP and Synaptophysin

Immunostaining of NACP and synaptophysin bands was carried out using anti-NACP(131–140) and mouse monoclonal antibody SY38, respectively. Signal intensity was quantified by scanning with a densitometer. Each value is shown as a relative amount of protein normalized to the value in frontal cortex. NACP is highly concentrated in olfactory bulb, frontal cortex, striatum, and hippocampus, whereas synaptophysin is distributed more uniformly throughout the brain.

of a group of closely related proteins, all of which may be detected by the anti-NACP antibody that we employed (Jakes et al., 1994).

NACP was found to be distributed with a unique pattern in the brain, whereas synaptophysin was found to be distributed more ubiquitously. Both immunohistochemical and biochemical data demonstrated that NACP is relatively abundant in the telencephalon, including the olfactory bulb, cerebral cortex, striatum, and hippocampus, and is expressed moderately in the thalamus. NACP was reported by Uéda et al. (1993), using Northern blot analysis, to be produced mainly in the central nervous system. Together, these data suggest that NACP might have some characteristic function in the telencephalon, which may be acquired during brain development.

In the central nervous system, APP, the precursor of the major amyloid component Aβ, is found in neurons, glia, and ependymal and mantle cells (Card et al., 1988; Kawarabayashi et al., 1991). This protein is reported to be localized in the cell body, especially on or near the plasma membrane (Shivers et al., 1988), in Golgi (Caporaso et al., 1994), or transported to a synaptic site by fast anterograde axonal transport (Koo et al., 1990; Schubert et al., 1991). Differing from the distribution of APP, NACP was found in close vicinity of the synaptic vesicles in presynaptic terminals, but not in the cell body of neurons. This distribution was similar to that of synaptophysin identified by a mouse monoclonal antibody, SY38 (Wiedenmann and Franke, 1985). Synaptophysin is a synaptic vesicle protein used as a "presynaptic marker" (Masliah et al., 1991b, 1992a). Double immunostaining with anti-synaptophysin suggests that NACP is also specific to presynaptic terminals and may have a role for presynaptic function, involving synaptic vesicles. Because synaptophysin is an integral membrane protein of synaptic vesicles, it is detected in the particulate fraction in Western blot. On the other hand, NACP was found mainly in the cytosolic fraction. Therefore, it is possible that NACP is loosely associated with the membrane or membrane-binding proteins of synaptic vesicles as a peripheral protein like F1-ATPase on the mitochondrial inner membrane (Horstman and Racker, 1970). The hydrophobic domain of NACP including the NAC portion might be the region responsible for this putative association with the synaptic vesicles.

NACP showed high homology with the human synuclein β, rat synucleins, Torpedo synuclein, and bovine PNP-14. Synucleins and PNP-14 are neuron-specific proteins found to be concentrated in presynaptic terminals (Maroteaux et al., 1988; Maroteaux and Scheller, 1991; Nakajo et al., 1993; Jakes et al., 1994). Two additional human proteins, the identities of which are not known, were also found to show homology with NACP. The existence of several alternatively spliced forms and other homologous sequences suggests that NACP, synucleins, and PNP-14 are members of a protein family. In previous reports synucleins were found not only in presynaptic terminals but

Figure 4. Laser Scanning Confocal Microscopy of Sections Double-Labeled for SY38 and NACP(131–140)

Mouse monoclonal anti-synaptophysin (SY38) is labeled with fluorescein isothiocyanate and shown in green (A, D, and G); NACP(131–140) is labeled with Texas red (B, E, and H). (C), (F), and (I) correspond to the electronically merged image; colocalization of NACP with synaptophysin is represented in yellow. (A–C) Mossy fiber terminals in the hippocampal CA3; (D–F) nucleus pontis; (G–I) dentate nuclei of the cerebellum. Bar, 15 μm.

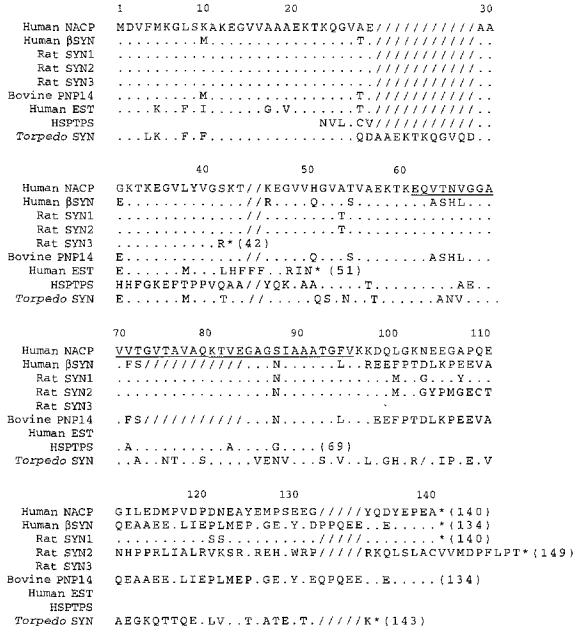


Figure 7. NACP Sequence Homologies

Number refers to the amino acids from the first methionine of NACP. The NAC portion found in the AD amyloid is underlined. Identical amino acids among proteins are indicated by dots. Insertions (/) were introduced to maximize the homology. Only different amino acids were shown in the proteins, except for NACP. βSYN, β-synuclein; SYN1, synuclein 1; SYN2, synuclein 2; SYN3, synuclein 3; bovine PNP-14, bovine phosphoneuroprotein 14; Human EST, EST01420 found by random sequencing of human brain cDNAs (Adams et al., 1992); HSPTPS, Homo sapiens putatively transcribed partial sequence found by UK-HGMP; Torpedo SYN, Torpedo synuclein.

also in nuclei in Torpedo, the reason why the proteins bear the name "synuclein." However, our affinity-purified antibodies revealed that rat NACP is distributed only in presynaptic terminals, not in nuclei. PNP-14 was also reported to be localized uniquely to presynaptic terminals. The reasons for these different results may reside in the differences of the antisera and the animals. The antiserum recognizing both the nuclear and synaptic proteins was raised using the fusion protein of Torpedo synuclein–β-galactosidase and was used without affinity purification. This antiserum might have cross-reactivity with the other Torpedo proteins in the nucleus. In fact, this antiserum against "synuclein" did not stain a single band on a Western blot of crude homogenate. On the other hand, some isoforms of synuclein might be distributed in nuclei specifically in the fish. Our antisera, anti-NACP(1–9) and anti-NACP(131–140), were raised using the synthetic peptides of the human NACP N- and C-terminal sequences and purified with the corresponding peptides. On Western blots, our affinity-purified antibodies recognized uniquely the bands corresponding to NACP in both rat and human brain homogenate. The data with our specific antibodies suggest that NACP is a presynaptic protein in the mammalian brain, and the name "synuclein" may not be appropriate for this protein.

It has been reported that presynaptic damage and structural abnormalities are found in the AD brain, with the most pronounced changes in senile plaques, and that this synaptic damage correlates well with cognitive impairment (Terry et al., 1991; Masliah et al., 1992a). The relationship between synaptic damage within senile plaques and amyloidogenesis is not clear. It is noteworthy that the components located at the synaptic site are associated with neuritic alteration and plaque formation in AD. APP, the precursor of the major constituent of AD amyloid, accumulates in dystrophic neurites in a subset of senile plaques (Shoji et al., 1990; Cole et al., 1991; Joachim et al., 1991; Masliah et al., 1992b). Chromogranin A, a marker protein for the large dense-core vesicles in presynaptic terminals, and synaptophysin were both found in dystrophic neurites of AD senile plaques, and the ratio of chromogranin A to synaptophysin was increased in AD (Weiler et al., 1990; Brion et al., 1991; Munoz, 1991; Lassmann et al., 1992). GAP-43, a marker protein for both sprouting presynaptic terminal and growth cones, also accumulates in the dystrophic neurite in senile plaques (Masliah et al., 1991b). However, peptide fragments derived from these presynaptic proteins were not detected in the amyloid, except for APP and NACP, possibly reflecting the absence and presence of an "amyloidogenic" sequence within these proteins. In addition to amyloid and neuronal components, both astroglial and microglial cells are another prominent feature of senile plaques (Price et al., 1992). Glial components participate in the formation of plaques only at the late steps, and they are not found in diffuse plaques, the most primitive stage of plaques. As both Aβ and NAC are immunopositive in diffuse plaques, it is conceivable that metabolically abnormal presynaptic terminals which contain APP and NACP are already associated with diffuse plaques. Thus, synaptic abnormality might be closely related to the early stage of amyloidosis and plaque formation. Investigation of NAC and NACP may help elucidate further the molecular mechanisms of amyloidosis and synaptic alteration, and their interactions in AD brain.

Experimental Procedures

Antibodies against NACP

Two peptide sequences, 9 amino acid N-terminal NACP (MDVFMKGLS) and 10 amino acid C-terminal NACP (EGYQDYEPEA), were synthesized, with an additional cysteine at each C-terminus for conjugation with the carrier protein keyhole limpet hemocyanin (Calbiochem, San Diego, CA; Green et al., 1982), by a Rainin PS3 peptide synthesizer (Rainin, Woburn, MA) or an Applied Biosystems peptide synthesizer (Model 430A, Applied Biosystems, Foster City, CA) and purified by a Waters high performance liquid chromatograph (Model 600E, Waters, Milford, MA) with a YMC Pack R & D D-ODS column using 0%–70% acetonitrile including 0.1% trifluoroacetic acid. Each peptide (10 mg) was conjugated with keyhole limpet hemocyanin (10 mg) in 2 ml of phosphate-buffered saline (PBS; 0.01 M phosphate, 0.15 M NaCl [pH 7.4]) by 2.5 mg/ml m-maleimidobenzoyl-N-hydroxysuccinimide ester for 30 min with stirring and dialyzed overnight against PBS using dialyzing tubing with a 5000–6000 Da cutoff to remove uncoupled peptide. A 200 μg quantity of peptide, emulsified in Freund's complete adjuvant, was administered subcutaneously to New Zealand rabbits, and 100 μg of peptide emulsified in Freund's incomplete adjuvant was used subsequently several times at 2–3 week intervals for boosting. Antibodies were purified using affinity chromatography with immobilized corresponding peptide. Pierce Immuno-Pure Ag/Ab immobilization kit #1 (Pierce, Rockford, IL) was used for

antibody purification following the company-recommended protocol. Briefly, 1 mg of peptide was coupled to activated agarose in 0.1 M sodium cyanoborohydride. The 50% ammonium sulfate precipitate from 5 ml of serum was incubated with this affinity agarose overnight at 4°C. After several washings with PBS, specific antibody was eluted by 0.1 M glycine/HCl (pH 2.8). The protein-containing fractions were immediately neutralized with 1 M Tris–HCl (pH 9.5), and the protein peaks were pooled and dialyzed against PBS using dialyzing tubing with a 5000–6000 Da cutoff. The purified antibody raised to NACP (1–9) was named "anti-NACP(1–9)" and the one to NACP(131–140) was named "anti-NACP(131–140)."

Bacterially Expressed Human NACP

The bacterial NACP expression construct, pSENACP, was previously described (HBS6–1; Uéda et al., 1993). The E. coli HB101 transformant harboring pSENACP was cultured in Luria–Bertani medium to mid-log phase, and isopropyl-1-thio-β-D-galactoside was added to a final concentration of 0.5 mM and cultured for 4 hr. Cells were harvested by centrifugation and disrupted by sonication. The lysate was centrifuged at 100,000 × g at 4°C for 30 min to recover the supernatant that contained the NACP protein. The purity of the NACP protein in this preparation was about 10%.

Specificity of Antibodies in Western Blot

Western blot procedures were described previously (Masliah et al., 1991a). Briefly, human midfrontal cortex gray matter and young adult Sprague–Dawley rat frontal cortex were homogenized in 10 vol of homogenizing buffer (5 mM HEPES [pH 8.0], 0.32 M sucrose, 5 mM benzamidine, 2 mM β-mercaptoethanol, 3 mM EGTA, 0.5 mM $MgSO_4$, 0.05% NaN_3) containing protease inhibitors (10 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (10 μM sodium orthovanadate, 2 mM KF, 1 μM okadaic acid) using a Teflon/glass homogenizer at 4°C. Homogenized samples were centrifuged at 100,000 × g for 1 hr at 4°C. The supernatant was used as the cytosolic fraction, and the rehomogenized pellet in the original volume of homogenizing buffer was used as the particulate fraction. The soluble fraction of E. coli transfected with pSENACP was used as an "authentic" human NACP. As a negative control, the soluble fraction of the E. coli homogenate transfected with pSE380 was used (vector control). Protein concentration was determined by Bradford assay (Bio-Rad protein assay, Bio-Rad, Hercules, CA) with a protocol recommended by the manufacturer. Protein from brain homogenate (40 μg) and protein from E. coli homogenate (1.5 μg; containing about 150 ng of NACP) in Laemmli sample buffer (Laemmli, 1970) were electrophoresed on 16% SDS–polyacrylamide gels and blotted to nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). The membrane was incubated in PBS with 0.3% Tween-20 for 1 hr at room temperature to block nonspecific binding sites. The affinity-purified antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and 0.1% Tween-20 at the dilution of 1:100–1:30. The membrane was incubated with the antibody solution at 4°C overnight, washed in PBS containing 0.1% Tween-20, incubated with 0.5 μCi/ml iodinated protein A (ICN Pharmaceutical, Inc., Covina, CA) in PBS containing 3% BSA and 0.1% Tween-20 for 1 hr, washed, wrapped, and apposed to Kodak X-Omat RP film at −80°C. Films were then developed with a Konica film developer. The specificity of each antibody was confirmed by the absence of a NACP band in Western blots incubated with antibody preabsorbed with each corresponding peptide with a ratio of 5 μg peptide to 1 μl of purified antibody.

Immunocytochemistry and Laser Scanning Confocal and Immunoelectron Microscopy

Sprague–Dawley rats weighing 250–300 g were deeply anesthetized by sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) and perfused transcardially with PBS and then with 4% paraformaldehyde in PBS for 20 min, and the brain was removed and immediately placed in a postfixative, 4% paraformaldehyde solution, for 4 days at 4°C. The brain was immersed in a 30% sucrose solution in PBS for 4 days at 4°C, frozen in −40°C hexane, and cut into 20 μm sections using a cryostat at −20°C.

Immunohistochemistry was performed according to the modified SAB method. Sections were rinsed three times for 5 min each in PBS, incubated for 10 min in PBS containing 0.1% Triton X-100, and incubated for 20 min with 3% H_2O_2 to inhibit endogenous peroxidase. They were then incubated for 10 min with 10% normal goat serum (NGS; Nichirei, Tokyo, Japan) in PBS and incubated for 18 hr at 4°C with anti-NACP(1–9) or anti-NACP(131–140) antibody in PBS containing 1% BSA. Sections were rinsed three times for 5 min each in PBS and incubated with biotinylated goat anti-rabbit IgG solution (Nichirei, Tokyo, Japan) for 10 min. They were then rinsed three times for 5 min in PBS, incubated with streptavidin–peroxidase solution (Nichirei, Tokyo, Japan) for 5 min, and rinsed three times for 5 min each in PBS. NACP-positive structures were visualized by incubating the tissue in 0.05% diaminobenzidine (DAB) with 0.01% H_2O_2 in 0.61 M Tris–HCl buffer (pH 7.4) for 5–15 min. Specificity of the immunohistochemical reaction was confirmed by the absence of staining in adjacent tissue sections incubated with antibody preabsorbed as previously described (Uéda et al, 1993).

Double immunostaining was performed with antibodies against NACP and synaptophysin as described previously (Masliah et al., 1991b). After treatment with 0.1% Triton X-100 and 3% H_2O_2, sections were incubated for 1 hr with 5% normal horse serum (Vector Labs, Inc., Burlingame, CA), 10% NGS (Vector Labs, Inc.), and 2% BSA in PBS. They were then incubated for 18 hr at 4°C with the mixture of mouse monoclonal antibody against synaptophysin (SY38, Boehringer Mannheim, Indianapolis, IN; Wiedenmann and Franke, 1985) and rabbit polyclonal antibodies, anti-NACP(1–9) or anti-NACP(131–140), in PBS containing 3% normal horse serum, NGS, and BSA. Sections were rinsed three times for 5 min each in PBS, incubated for 1 hr with biotinylated goat anti-rabbit IgG in PBS containing 1% BSA, and rinsed three times for 5 min each in PBS. They were then incubated for 80 min with Texas red–labeled avidin (Vector Labs, Inc.) and fluorescein isothiocyanate–labeled horse anti-mouse IgG antibody (Vector Labs, Inc.) in PBS containing 1% BSA, and rinsed three times for 5 min each in PBS. The double-immunolabeled sections were covered with glass coverslips with antifading medium (Vector Labs, Inc.). These sections were imaged with the Bio-Rad MRC-600 laser confocal scanning microscope mounted on a Zeiss Axiovert microscope (Masliah et al., 1991a, 1992b). This system permits the simultaneous analysis of double-labeled samples in the same optical plane. The digitized video images of serial 0.5 μm optical sections were stored on an optical disk for subsequent processing and analysis. Electronically merged digital images were analyzed with the Image 1.43 program (Wayne Rasband, NIH) to calculate the percentage of presynaptic terminals that contain either one or both of the markers (Masliah et al., 1991b).

For electron microscopic study of anti-NACP staining detected by DAB, 40 μm vibratome sections were blocked with NGS (5%) and incubated overnight at 4°C with anti-NACP(131–140) (Masliah et al., 1991a). The sections were washed in PBS, incubated with biotinylated goat anti-rabbit IgG followed by avidin D-HRP (Vector ABC elite, Vector Labs, Inc.), and reacted with DAB (0.2 mg/ml) in 50 mM Tris–HCl buffer (pH 7.4) with 0.001% H_2O_2. To prevent the artifactual diffusion of the chromogen, the sections were incubated in 1% glutaraldehyde in 0.15 M cacodylate buffer before the DAB. Control experiments were performed by incubating sections with nonimmune serum. The immunostained sections were postfixed for 20 min in 1% OsO_4, dehydrated, and flat embedded in epoxy/Araldite. Ultrathin sections were cut with a Leica Ultracut E ultramicrotome and viewed with a 100 CX JEOL electron microscope.

The vibratome sections for immunogold staining were embedded in Durcupan, and ultrathin sections were cut as above. The sections were treated with 1% $NaIO_4$ in water, 50 mM glycine in PBS, and 1% BSA in PBS, each for 4 min with rinsing in water between each step. Sections were incubated with anti-NACP(131–140) antibody as above, rinsed with 1% BSA in PBS, incubated with a 1:30 dilution of 5 nm gold goat anti-rabbit IgG (Amersham) for 1 hr, and rinsed again. The immunogold-stained sections were postfixed with 1% glutaraldehyde in PBS for 3 min and counterstained with uranyl acetate and lead citrate before examination with an electron microscope as above.

Biochemical Quantification of NACP and Synaptophysin

Brains of young adult Sprague–Dawley rats weighing 250–300 g were separated into ten portions as follows: olfactory bulb, frontal cortex, striatum, hippocampus, hypothalamus, thalamus, midbrain, cerebellum, pons/medulla oblongata, and pituitary. Each portion was homoge-

nized as described earlier and used in Western blots for NACP and synaptophysin quantification. Protein (40 μg) from the particulate fraction (for synaptophysin quantitation) or from the cytosolic fraction (for NACP quantitation) was loaded on a 10% SDS–polyacrylamide gel and blotted to nitrocellulose membrane. The rabbit anti-NACP(131–140) antibody in PBS containing 1% BSA and 0.1% Tween-20 was used for NACP quantitation. For synaptophysin quantitation, the mouse monoclonal antibody against synaptophysin (SY38, Boehringer Mannheim), in PBS containing 1% BSA and 0.1% Tween-20, was used as the primary antibody, followed by rabbit anti–mouse IgG polyclonal antibody (Accurate Chemical and Scientific Corp., Westbury, NY) at the dilution of 1:2000 in PBS containing 3% BSA. After incubation with antibodies, blots were rinsed, incubated with 0.5 μCi/ml iodinated protein A, rinsed further, and apposed to Kodak X-Omat RP film at −80°C. Films were then developed with a Konica film developer and scanned with an LKB densitometer for quantitation of the NACP or synaptophysin bands. The exposure time was chosen so that the intensity of bands were in the OD range between 0.8 and 2.5, where the sensitivity of the film is relatively linear.

NACP Homologies

Computer homology search (FASTA program) was used for searching the DNA sequence database (European Molecular Biology Laboratory/GenBank Libraries). Also, the BLAST network service at NCBI (National Center for Biotechnology Information, Bethesda, MD) was used to search for homologous sequences.

Acknowledgments

The authors thank Drs. Allen D. Roses, Kenji Uéda, and Tokio Yamaguchi for discussions and comments, Deborah A. C. Otero and Yu Xia for technical assistance, and Mary P. Sundsmo and Robert W. Davignon for editorial help. H. A. Rohan de Silva was supported by a fellowship from the Joseph Bryan Scholars Foundation. This work was supported by grants from the American Health Assistance Foundation and the NIH (AG05131 and AG10689). Yamanouchi Pharmaceutical Co., Ltd. offered technical and material help.

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Received May 25, 1994; revised October 18, 1994.

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